"Stealth Effect": Adenocarcinoma Cells Engineered to Express TRAIL Elude Tumor-Specific and Allogeneic T Cell Reactions[1]

Mirella Giovarelli,[2]* Piero Musiani,† Gianni Garotta,§ Reinhard Ebner,§ Emma Di Carlo,† Yunsoo Kim,§ Paola Cappello,* Laura Rigamonti,* Paola Bernabei,* Francesco Novelli,* Andrea Modesti,‡ Anna Coletti,‡ Ann Kim Ferrie,§ Pier-Luigi Lollini,* Steve Ruben,§ Theodora Salcedo,§ and Guido Forni*

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It has recently been reported that activated monocytes mediate their tumoricidal activity via the membrane expression of TRAIL (9). Moreover, systemic administration of TRAIL trimers induces apoptosis of many cancer lines without the deleterious side-effects of CD95 ligand (CD95L) or TNF (10). Since TRAIL appears to rapidly exert potent antitumor activity by selectively and directly activating tumor cell apoptosis (8–11), this finding opens intriguing new perspectives in the treatment of cancer (4, 11). However, the TNF family members are involved in a basic regulation of immune responses by biasing the action of T cells that encounter Ag toward either activation (12) or apoptotic (13–15) pathways. This paper reports that mouse mammary adenocarcinoma cells (TSA) (16) engineered to express TRAIL on their membrane (TSA-TRAIL) are better suited to overcome tumor-specific immunity and grow across minor and major histocompatibility barriers than wild-type parental cells (TSA-pc). The eventual rejection of TSA-TRAIL cells by allogeneic mice does not result in a significant boosting of a specific immune memory, since TSA-TRAIL cells appear to induce apoptosis of activated T lymphocytes.

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BALB/c mammary adenocarcinoma cells engineered to express TNF-related apoptosis-inducing ligand (TRAIL)/APO-2 ligand (APO-2L) on their membrane (TSA-TRAIL) grow with kinetics similar to that of parental cells (TSA-pc) in vitro and in *nu/nu* mice. In contrast, TSA-TRAIL cells grow faster than TSA-pc in normal BALB/c mice. In DBA/2 mice, which differ from BALB/c mice at minor histocompatibility Ags, they also grow faster and display a higher percentage of tumor takes than TSA-pc. In fully histoincompatible C57BL/6 (B6) mice, TSA-TRAIL cells form evident tumors that are slowly rejected by most mice, but outgrow in a few. In contrast, TSA-pc cells are rejected at once by B6 mice. Since TRAIL/APO-2L induces apoptosis by interacting with a variety of specific receptors, this rapid growth in both syngeneic and allogeneic mice may be the result of an immunosuppressive mechanism. The following evidence supports this hypothesis: 1) TSA-TRAIL cells overcome the strong immunity against TSA-pc cells elicited in BALB/c mice by preimmunization with TSA cells engineered to release IL-4; 2) their rejection by B6 mice does not prime a CTL-mediated memory; 3) thymidine uptake by T lymphocytes unstimulated or stimulated by allogeneic cells is inhibited when TSA-TRAIL cells are added as third party cells; 4) CTL kill TSA-pc but not TSA-TRAIL cells in 48-h assays; and 5) activated lymphocytes interacting with TSA-TRAIL cells in vivo and in vitro undergo apoptosis. *The Journal of Immunology*, 1999, 163: 4886–4893.

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(BioWhittaker, Walkersville, MD), and then suspended in OptiMEM without FBS cells and transfected with 10 μg pCDNA3-TRAIL DNA using LipofectAMINE reagent (Life Technologies, Rockville, MD). Forty-eight hours after transfection, the cells were split at a ratio of 1:10 and plated in DMEM medium with 1 mg/ml of G418 (Schering-Plough, Milan, Italy). Clones were isolated and subcloned by limiting dilution 15–20 days later. TSA-neo is a control clone transfected with the neomycin resistance gene only.

Anti-TRAIL Abs

TRAIL cDNA was expressed in *Escherichia coli*. Rabbits were immunized with 0.5 mg of renatured TRAIL protein obtained from bacterial pellets and purified by chromatography on a nickel-NTA chelate column. The anti-TRAIL IgG were affinity purified on protein A-Sepharose (Amersham, Pharmacia Biotech, Uppsala, Sweden).

In vitro cultures

In vitro cultures were performed with sterile disposable glassware from Nunc, Roskilde, Denmark, at 37°C in a humidified atmosphere with 5% CO_2, using RPMI 1640 medium (BioWhittaker, Milan, Italy) supplemented with 10% FBS, 50 μg/ml gentamicin, 2.5×10^{-5} M β_2-ME (Sigma, Milan, Italy), except when otherwise specified.

Tumor

TSA-pc are a cell line from a moderately differentiated mammary adenocarcinoma that arose spontaneously in a BALB/c mouse (16). TSA-pc express MHC class I, but not class II, molecules, secrete G- and GM-CSF and TGF-β1, and do not stimulate a syngeneic antitumor response in vivo or in vitro (16, 18, 19). Similar features are displayed by TSA-TRAIL (data not shown). F1-F is an in vitro-transformed newborn BALB/c mouse-derived skin fibroblast line that does not immunologically cross-react with TSA-pc (20). Inocula of 5×10^4 and 10^4 cells are about the minimal 100% TSA-pc and F1-F tumor-inducing doses in BALB/c mice. In a few experiments, mice were preimmunized against TSA-pc by challenging them in the right flank with 10^5 living TSA cells engineered to release 40 U of IL-4/10^5 cells/ml in 48 h incubation (20). Those without tumor 1 mo after challenge were used as TSA-pc-immune mice. P815 mastocytoma (H-2^d) and EL-4 (H-2^b) lymphoma were cultured as nonadherent cell cultures.

Mice

Seven-week-old female *nu/nu* (CD1), BALB/c (H-2^d), DBA/2 (H-2^d), C57BL/6 (B6) (H-2^b), and C3H (H-2^k) (Charles River Laboratories, Calco, Italy) were treated in accordance with the European Union guidelines. When required, starting 2 days before tumor challenge and 4 h after, and then weekly, a few mice received i.p. injections of 100 μg anti-CD8 (TIB-105 hybridoma, American Type Culture Collection, Manassas, VA) or normal rat Ig purified through an anionic exchange column (DE 52, Whatman, Maidstone, England). Cytofluorimetric analysis of the residual blood and spleen cells from mice receiving these Abs showed that target leukocytes were selectively decreased to 1/5000 of peripheral blood leukocytes during treatment.

In vivo evaluation of tumor growth

Mice were challenged s.c. in the left flank with 0.2 ml of a single cell suspension containing the indicated number of tumor cells. The cages were coded, and neoplastic masses were measured with calipers in the two perpendicular diameters twice weekly for 120 days in a blind fashion. Tumor-free mice at the end of this period were classed as survivors. Latency and survival times were considered as the periods (in days) between challenge and the growth of neoplastic masses of 3 and 10 mm mean diameter, respectively. Only mice that eventually developed tumor were considered. Mice were killed for humane reasons when the tumor exceeded 10 mm mean diameter.

Morphological analysis

For histological evaluation, tissue samples from groups of five mice were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 4 μm, and stained with hematoxylin and eosin or Giemsa. For immunohistochemistry, acetone-fixed cryostat sections were incubated for 30 min with the following Abs: anti-MAC-1, anti-MAC-3, anti-I-A/I-E, anti-IL-6, anti-VCAM-1 (PharMingen, San Diego, CA); anti-granulocytes (RB6-8C5, kindly provided by Dr. Coffman, DNAX, Palo Alto, CA); anti-CD4, anti-CD8 (Sera Laboratories, Crawley Down, Sussex, U.K.); anti-IL-1β (Genzyme, Cambridge, MA); anti-TNF-α (Immuno Kontact, Frankfurt, Germany); anti-IFN-γ (kindly provided by Dr. Landolfo, Torino University, Italy); anti-inducible NO synthetase (iNOS) (Transduction Laboratories, Lexington, Ky); anti-CD31, anti-CD62 (kindly provided by Dr. Vecchi, Negri Nord, Italy); anti-ICAM-1 (CD54, Santa Cruz Biotecnology, Santa Cruz, CA). After washing, the sections were overlaid with biotinylated goat anti-rat, anti-hamster, and anti-rabbit and horse anti-goat Ig (Vector Laboratories, Burlingame, CA) for 30 min. Unbound Ig was removed by washing, and the slides were incubated with avidin-biotin complex/alkaline phosphatase (Dako, Glostrup, Denmark). Quantitative studies of the immunohistochemically stained sections were performed by three pathologists in a blind fashion on three or more samples from distinct mice by evaluating ten randomly chosen fields in each sample. For cell counts, individual cells were counted under a microscope (×40 objective and ×10 ocular lens; 0.18 mm^2 per field). The expression of cytokines and adhesion molecules was defined as absent (−) or as scarcely (+), moderately (++), or frequently (+++) present on cryostat sections tested with the corresponding Ab. For electron microscopy, specimens were fixed in cacodylate buffered 2.5% glutaraldehyde, postfixed in osmium tetroxide, and then embedded in Epon 812. Ultrathin sections were stained with uranyl acetate-lead citrate.

Flow cytometry

TSA-TRAIL and TSA-pc from tumors grown in vivo were incubated with a 1:10 dilution in HBSS-azide-BSA of normal mouse Ig or mouse mAb to H-$2K^d$ (clone 31-3-4S) and H-$2D^d$ (clone 34-5-8S) (Cedarlane, Hornby, Ontario, Canada) or to H-$2L^d$ (clone 28-14-8S) (Litton Bionetics, Charleston, SC) followed by incubation with FITC-F(ab')$_2$ goat anti-mouse Ig (Technogenetics, Milan, Italy). Cultured TSA-TRAIL and TSA-pc were incubated with 1:10 dilution of normal rabbit IgG or anti-TRAIL rabbit IgG followed by incubation with PE-F(ab')$_2$ goat anti-rabbit Ig (Biosource, Camarillo, CA). Dead cells were gated on the basis of forward and sideways scatter. All labeling steps were followed by incubation for 30 min at 4°C, and separated by two washes with HBSS-azide-BSA. In each

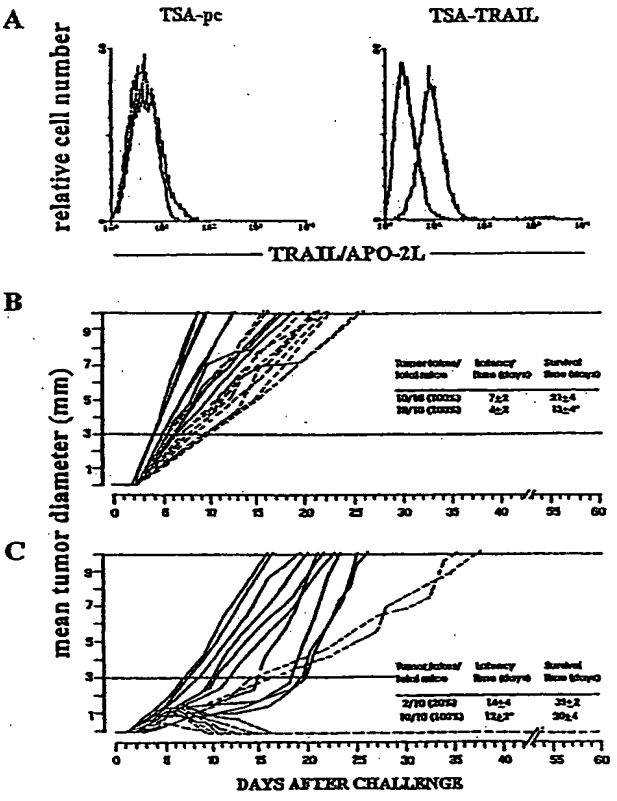

FIGURE 1. Characteristics of TSA-TRAIL cells. *A*, Expression of TRAIL on the membrane of TSA-pc (3% positive; mean 2; median 0) and TSA-TRAIL cells (71% positive; mean 18; median 6). Cells were incubated with specific anti-TRAIL/APO-2L rabbit IgG (bold lines) or control normal rabbit IgG (thin lines) and then with PE-F(ab')$_2$ goat anti-rabbit Ig. *B and C*, Growth and rejection patterns of 10^6 (*B*) or 10^5 (*C*) TSA-TRAIL (solid line) and TSA-pc (dashed line) injected s.c. in the left flank of DBA/2 mice. Mice were surveyed for 120 days after challenge.

Table I. *Comparison of the growth of TSA-pc and TSA-TRAIL cells in T-deficient nu/nu mice and in immunocompetent BALB/c mice syngeneic with TSA tumor*

Recipient Mice	Challenging Cells: Type	Challenging Cells: Dose	Anti-CD3 mAb	Tumor Takes/ Total Mice	Latency Time (days)	Survival Time (days)
nu/nu	TSA-pc	1×10^5	−	4/4	9.0 ± 3.0	18.0 ± 1.6
nu/nu	TSA-TRAIL	1×10^5	−	4/4	7.5 ± 1.7	18.0 ± 2.9
BALB/c	TSA-pc	1×10^6	−	30/30	7.5 ± 1.8	16.5 ± 3.5
BALB/c	TSA-TRAIL	1×10^6	−	22/22	2.2 ± 2.2[a]	13.1 ± 4.1
BALB/c	TSA-pc	1×10^5	−	35/35	12.3 ± 2.5	23.1 ± 5.8
BALB/c	TSA-pc	1×10^5	+	10/10	8.0 ± 1.2[b]	17.4 ± 1.1[b]
BALB/c	TSA-TRAIL	1×10^5	−	22/22	7.9 ± 1.5	16.9 ± 2.8[a]
BALB/c	TSA-TRAIL	1×10^5	+	10/10	6.3 ± 0.6	16.7 ± 1.1

[a] Values significantly different from those of mice challenged with the same dose of TSA-pc.
[b] Values significantly different from that of nonimmunosuppressed mice.

experiment, 10^4 viable cells were analyzed with a FACScan flow cytometer (Becton Dickinson, Mountain View, CA).

Mixed lymphocyte reactions

MLR were performed either in triplicate in the wells of 96-well, flat-bottom plates or in 75-cm^2 culture flasks with culture medium supplemented with 4% FBS only as previously described in detail (20). Responder nylon wool effluent spleen cells (>90% Thy 1.2^+) or mesenteric lymph node cells (4×10^5/well or 25×10^6/flask) were cultured for 5 days either alone or with the same number of allogeneic spleen cells pretreated with mitomycin C. In a few cases, progressive numbers of mitomycin C-pretreated TSA-TRAIL or TSA-pc were added at the beginning of the cultures as third party cells. Each well was pulsed with 1 μCi of [^{3}H]TdR for 8 h before harvesting. [^{3}H]TdR uptake was assayed in a liquid scintillation spectrophotometer, and the data were expressed as Δcpm.

Cellular cytotoxicity

Cytotoxicity was evaluated in a 4-h sodium [^{51}Cr]chromate or 48-h [^{3}H]TdR release assay by mixing in triplicate various concentrations of effector lymphocytes with 5×10^3 labeled target cells at 50:1, 25:1, 12:1, and 6:1 E:T ratios as previously described in detail (20). The results were expressed as lytic units$_{20}$/10^7 cells (LU_{20}), calculated according to the equation of Pross et al. (20).

Apoptosis TUNEL analysis

Apoptosis was evaluated by fluorochrome labeling of DNA strand breaks by means of the TdT assay, using the Apo-Direct kit from PharMingen. This procedure allows the detection of apoptotic cells simultaneously with their DNA content (21). Early apoptotic cells can be detected by uptake of FITC-dUTP only, while later apoptotic cells can be detected as a hypo-diploid population (21). B6 lymph node and spleen cells (10^6) stimulated by BALB/c spleen cells for 3 days in MLR were cocultured overnight on monolayers of 2×10^5 TSA-TRAIL or TSA-pc, in medium or in the presence of 50 μg/ml of anti-TRAIL IgG, 50 μM of Z-Val-Ala-Asp (*O*-methyl)-fluoromethyl ketone (ZVAD-fmk), or 100 μM Ac-Y-Val-Ala-Asp (*O*-methyl)-cloromethyl ketone (YVAD-cmk) (Calbiochem, San Diego, CA), two specific inhibitors of caspase-1, or 1% DMSO as control. ZVAD-fmk and Ac-YVAD-cmk were dissolved in DMSO to yield a 10-mM stock solution. Recovered lymphocytes (10^6) were washed twice with PBS supplemented with 0.2% BSA and 0.05% sodium azide and suspended in 0.5 ml of PBS. Five milliliters of 1% paraformaldehyde in PBS was added, and the suspension was placed on ice for 15 min. Cells were then washed twice, 5 ml of ice-cold 70% ethanol was added, and the samples were stored at −20°C until use. Each sample was incubated for 60 min at 37°C with TdT enzyme and FITC-dUTP in a reaction buffer. The cells were washed and resuspended in 1 ml of propidium iodide and RNase solution, and then incubated for 30 min at room temperature. Samples were analyzed by flow cytometry within 3 h of staining.

Statistical analysis

The significance of differences ($p < 0.01$) in tumor takes was evaluated by Pearson's χ^2 test, whereas those in survival and latency time and in vitro data were evaluated by a two-sample Student *t* test.

Table II. *Reactive cell content, cytokine, and adhesion molecules expressed at the tumor growth area 7 days after TSA-pc and TSA-TRAIL cell challenge of BALB/c, DBA/2 and B6 mice*

	BALB/c Mice		DBA/2 Mice		B6 Mice
	TSA-pc	TSA-TRAIL	TSA-pc	TSA-TRAIL	TSA-TRAIL
Reactive cells					
Macrophages	19.8 ± 4.1[a]	34.4[b] ± 8.3	77.1 ± 10.4	36.6[b] ± 7.0	57.7 ± 10.1
Granulocytes	13.9 ± 3.6	20.4 ± 14.2	31.3 ± 6.7	16.0[b] ± 3.9	58.7 ± 9.6
$CD8^+$ lymphocytes	4.7 ± 2.1	3.6 ± 1.9	58.5 ± 9.6	20.6[b] ± 4.1	48.0 ± 7.1
$CD4^+$ lymphocytes	0.3 ± 0.1	6.1 ± 2.3	9.2 ± 7.6	8.1 ± 2.1	26.6 ± 5.4
Endothelial adhesion molecules					
ICAM-1	+	+	+++	++	+++
VCAM-1	−	+	+++	+	+++
ELAM-1	−	+	++	−	++
Cytokines and mediators					
IL-1β	−	−	+	−	+
TNF-α	−	+	+++	++	+++
IFN-γ	−	−	++	−	++
IL-6	+	+	+++	++	+++
iNOS	−	−	++	+	+++

[a] Cell counts were performed at ×400 in a 0.180 mm^2 field on 10 randomly chosen fields/sample. Results are the mean ± SD of positive cells/field.
[b] Values significantly different from corresponding values in TSA-pc.
ELAM, endothelial leukocyte adhesion molecule.

Results

TSA-TRAIL and TSA-pc growth in vitro and in nu/nu *and BALB/c mice*

TSA-pc are scarcely immunogenic in syngeneic BALB/c (H-2^d) mice, where they grow aggressively to form a poorly differentiated mammary adenocarcinoma (16, 18). They spontaneously secrete TGF-β, vascular endothelial growth factor, and GM-CSF (18), like most human and mouse tumors (19), and are easy to engineer genetically. The growth pattern and the modulation of immunogenicity of TSA cells transfected with many distinct membrane molecules and cytokines have been studied in detail in various laboratories (see a database in Ref. 18). This background information, along with the availability of early in vitro passages of TSA cells stored in liquid nitrogen, has allowed straightforward comparisons (18, 22). The selected clone (B021129) of TSA-pc transduced with the cDNA-encoding human TRAIL (TSA-TRAIL) displays the mRNA specific for TRAIL in Northern analysis (not shown) and expresses surface TRAIL protein (Fig. 1*A*), while still spontaneously secreting similar amounts of GM-CSF, TGF-β, and vascular endothelial growth factor as the TSA-pc (not shown). TSA-pc, TSA-neo cells engineered with control construct only, and TSA-TRAIL cells showed similar in vitro doubling times without major apoptotic deaths during both the exponential growth phase and at cell confluence, as detected by the TUNEL technique. These data rule out the possibility that fratricidal killing due to TRAIL membrane expression significantly affects TSA-TRAIL growth in vitro.

Table III. *Different ability of TSA-TRAIL and TSA-pc to grow in BALB/c mice preimmunized against TSA cells*

Preimmunizing	Immunosuppressive Treatment	Challenging Tumor Cells: Kind of tumor	Challenging Tumor Cells: Tumor takes/total mice
None	None	TSA-pc	10/10 (100%)[a]
None	None	TSA-TRAIL	10/10 (100%)
None	None	F1-F	5/5 (100%)
TSA-IL4	None	TSA-pc	0/10 (0%)
TSA-IL4	Anti-CD8	TSA-pc	9/10 (90%)
TSA-IL4	None	TSA-TRAIL	10/10 (100%)
TSA-IL4	Anti-CD8	TSA-TRAIL	10/10 (100%)
TSA-IL4	None	F1-F	5/5 (100%)

[a] Percentage of tumor takes, in parentheses.

Data obtained with TSA-pc and TSA-neo never differed significantly, and only the former will now be shown.

Both TSA-pc and TSA-TRAIL cells grew with a similar kinetics in T lymphocyte-deficient *nu/nu* mice. However, TSA-TRAIL cells grew faster in immunocompetent BALB/c mice (Table I). When BALB/c mice were depleted of $CD8^+$ lymphocytes, TSA-pc grew faster and this difference disappeared. Morphological observations 3 days after challenge showed that TSA-TRAIL and TSA-pc formed a solid tumor invading the fibroadipose tissue.

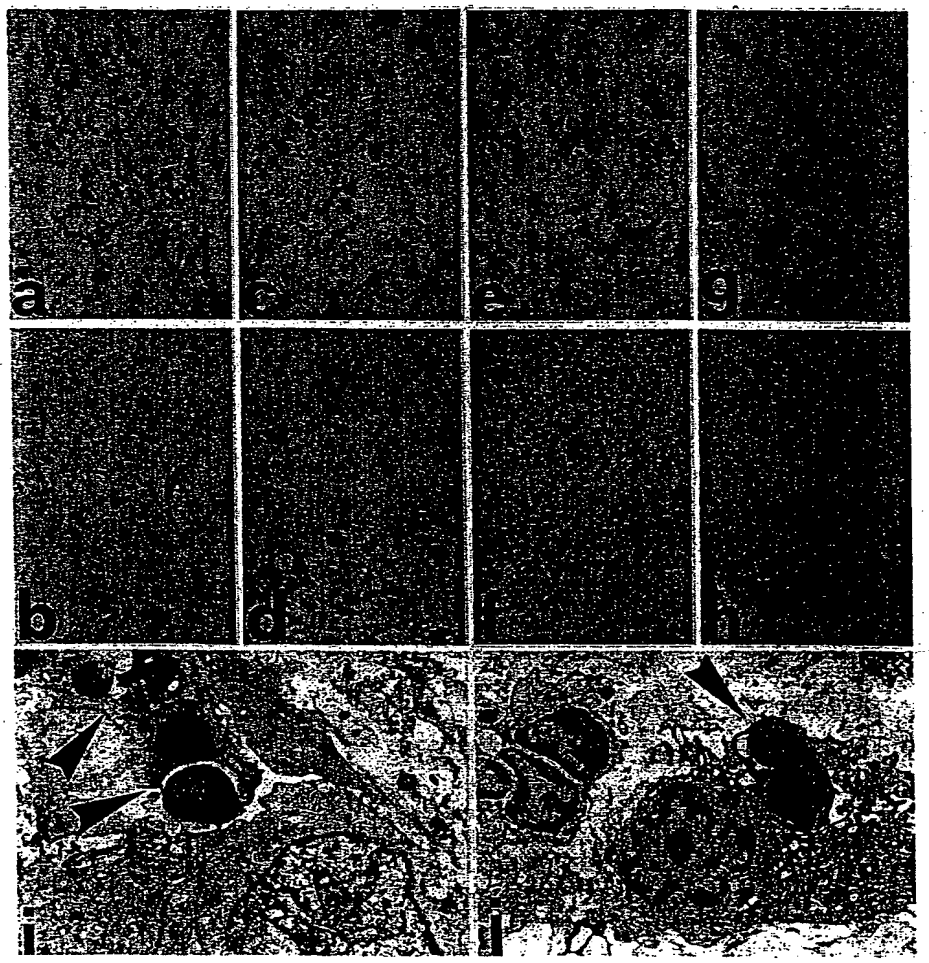

FIGURE 2. Immunohistochemical and ultrastructural aspects of TSA-TRAIL and TSA-pc 7 days after allotransplant in DBA/2 mice. Cryostat sections of TSA-pc (*upper* panel) and TSA-TRAIL (*middle* panel) tumors tested with anti-CD8 (*a* and *b*), anti-CD4 (*c* and *d*), anti-TNF-α (*e* and *f*), and anti-IFN-γ (*g* and *h*) mAb. Antilymphocyte staining shows that $CD8^+$ and $CD4^+$ cells are mainly located at the periphery of the tumor mass and less numerous in TSA-TRAIL (*b* and *d*) than in TSA-pc (*a* and *c*) tumor. The proinflammatory cytokines TNF-α and IFN-γ are less expressed in TSA-TRAIL (*f* and *h*) than in TSA-pc (*e* and *g*) tumor. Ultrastructural examination (*lower* panel) reveals that several TSA-TRAIL infiltrating lymphocytes show apoptotic changes: (*i*) apoptotic bodies (arrowheads) composed of dense convoluted cytoplasm with compacted nuclear fragment near a neoplastic cell; (*j*) a lymphocyte (arrowhead) in close contact with a neoplastic cell characterized by chromatin condensation into well-delimited dense masses under the nuclear membrane.

At the 7th day, TSA-TRAIL tumor masses were larger and displayed an evenly distributed vascularization without evidence of a major apoptotic death, nor the necrotic areas irregularly present in TSA-pc tumor. The reactive infiltrate of both tumors was scanty, and the macrophage and $CD4^+$ lymphocyte content of TSA-TRAIL tumor was significantly higher than that seen in the TSA-pc tumors (Table II). Immunohistochemistry revealed a slight expression of adhesion molecules by the well-developed vascularization of TSA-TRAIL tumor. Proinflammatory cytokines were poorly expressed in both tumors, though TNF-α was moderately expressed in TSA-TRAIL tumor (Table II).

Ability of TSA-TRAIL cells to overcome a tumor-specific immunity

Previous studies have suggested that TSA-pc are apparently nonimmunogenic in BALB/c mice. However, a challenge with TSA cells engineered to release cytokines is often rejected by most BALB/c mice and elicits the strongest TSA-specific immune response. The memory elicited by TSA-pc engineered to release IL-4 is among the strongest and is mostly based on TSA-specific $CD8^+$ T lymphocytes (22). Following TSA-IL-4 immunization, all mice rejected TSA-pc, whereas none of them rejected TSA-TRAIL cells (Table III).

TSA-TRAIL and TSA-pc tumorigenicity in allogeneic DBA/2 mice

One million TSA-TRAIL or TSA-pc always grew in DBA/2 mice, which have the same MHC as BALB/c mice, but differ at multiple minor histocompatibility Ags. However, TSA-TRAIL cells grew faster (Fig. 1*B*). A challenge of 10^5 TSA-TRAIL cells gave rise to progressively growing tumors in all DBA/2 mice, whereas the same dose of TSA-pc was rejected by most of them, and the progressively growing tumors grew more slowly (Fig. 1*C*).

Morphological evaluations 3 and 7 days after challenge showed that TSA-TRAIL tumor growth areas were almost similar to their counterparts in BALB/c mice, though some intratumoral necrotic areas were present. The TSA-pc tumor, on the other hand, was almost completely disaggregated by a massive reactive infiltrate in which macrophages and lymphocytes ($CD8^+ > CD4^+$) predominated (Fig. 2, *a* and *c*). The endothelial cells surviving from vascular damage strongly stained for adhesion molecules (not shown), while the whole TSA-pc growth area intensely stained for the proinflammatory cytokines TNF-α and IFN-γ (Fig. 2, *e* and *g*). In contrast, TSA-TRAIL tumors showed a scanty reactive infiltrate (Fig. 2, *b* and *d*), albeit with a distinct presence of lymphocytes associated with a moderate or scarce presence of adhesion molecules on the endothelial cells, and a limited expression of proinflammatory cytokines (Fig. 2, *f* and *h*). At day 7, lymphocytes interacting with TSA-TRAIL cells displayed ultrastructural features of cells undergoing apoptosis (Fig. 2, *i* and *j*).

TSA-TRAIL and TSA-pc tumorigenicity in B6 mice

B6 mice ($H\text{-}2^b$) differ from BALB/c mice at both multiple minor alloantigens and the full MHC. In these recipients, 10^6 TSA-pc were promptly rejected (Fig. 3*A*), whereas 10^6 TSA-TRAIL cells grew progressively in all mice and formed 4- to 9-mm tumor masses that later regressed in 79%. In three mice, TSA-TRAIL cells grew progressively and overcame the survival threshold. All mice rejected 10^5 TSA-pc cells, whereas a similar dose of TSA-TRAIL cells grew progressively, but overcame the survival threshold in two mice only (Fig. 3*B*). The unchanged expression of $H\text{-}2K^d$, $H\text{-}2D^d$, and $H\text{-}2L^d$ glycoproteins by TSA-TRAIL tumors in these mice shows that their ability to overcome histocompatibility barriers is not due to a reduction in MHC glycoproteins (Fig. 3*C*).

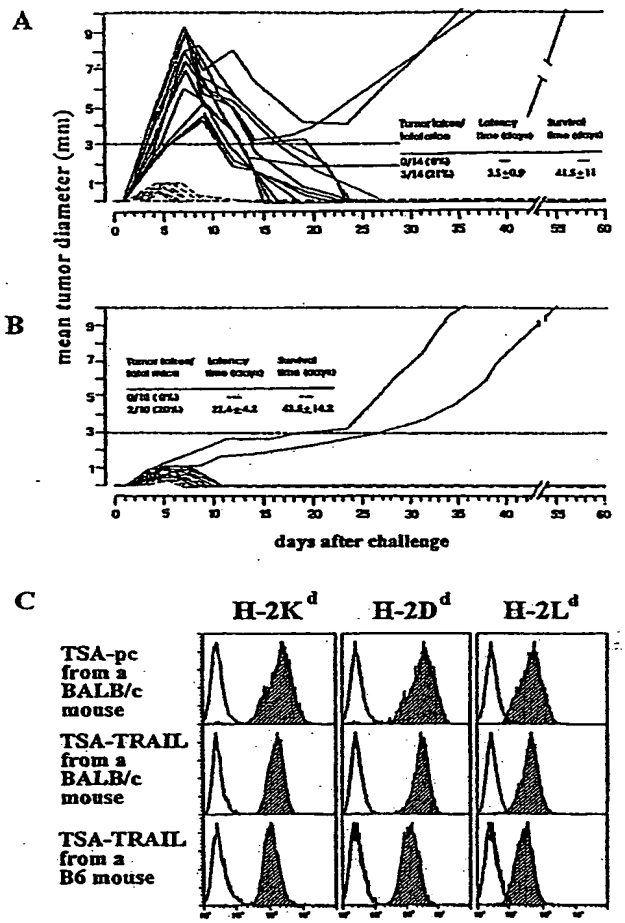

FIGURE 3. Growth and rejection patterns of 10^6 (*A*) or 10^5 (*B*) TSA-TRAIL (solid line) and TSA-pc (dashed line) injected s.c. in the left flank of B6 mice. Mice were surveyed for 120 days after challenge. *C*, Expression of H-2 alloantigens on TSA-TRAIL and TSA-pc from tumor grown in syngeneic BALB/c and in allogeneic B6 mice. When obtained from syngeneic BALB/c mice, TSA-pc were 99.8% positive for $H\text{-}2K^d$ (mean: 220; median: 270); 99.4% positive for $H\text{-}2D^d$ (mean: 352; median: 457); and 96.8% positive for L^d (mean: 49; median: 61). TSA-TRAIL were 99.7% positive for $H\text{-}2K^d$ (mean: 160; median: 187); 99.6% positive for $H\text{-}2D^d$ (mean: 294; median: 331); and 97.6% positive for L^d (mean: 44; median: 49). TSA-TRAIL cells recovered from a tumor grown in B6 mice were 99.8% positive for $H\text{-}2K^d$ (mean: 107; median: 130); 100% positive for $H\text{-}2D^d$ (mean: 129; median: 152); and 94.3% positive for L^d (mean: 28; median: 32).

Morphological evaluations showed that TSA-TRAIL cells elicited a leukocyte reaction. This was already evident on day 3 and became conspicuous on day 7. Granulocytes, macrophages, and $CD8^+$ lymphocytes were extremely numerous (Table II). Adhesion molecules were strongly expressed by blood vessels. An intense positivity for proinflammatory cytokines and iNOS was also detected. Three days after challenge, the reactive infiltrate invading the TSA-pc inoculum area was so massive that only a few scattered TSA-pc cells could be observed close to areas of ischemic-coagulative necrosis. This quick rejection prevented counting of the infiltrating cells and evaluation of cytokine expression. The lytic response to $H\text{-}2^d$ alloantigens primed by TSA-TRAIL and

Table IV. *Cytotoxicity of T lymphocytes from lymph nodes and spleens of B6 mice primed by TSA-TRAIL or TSA-pc*

	Responder B6 Lymphocytes from[a]	Stimulator Spleen Cells	LU_{20} Against P815 (H-2^d)	LU_{20} Against EL-4 (H-2^b)	LU_{20} Against PHA blasts (H-2^b)
Expt. 1	Lymph nodes of virgin mice	BALB/c	137	12	NT
	Lymph nodes of mice challenged with TSA-pc	BALB/c	788	14	NT
	Lymph nodes of mice challenged with TSA-TRAIL cells	BALB/c	290	12	NT
Expt. 2	Lymph nodes of virgin mice	BALB/c	591	18	12
	Lymph nodes of mice challenged with TSA-pc	BALB/c	875	21	15
	Lymph nodes of mice challenged with TSA-TRAIL cells	BALB/c	582	20	10
	Lymph nodes of virgin mice	C3H	132	NT	840
	Lymph nodes of mice challenged with TSA-pc	C3H	202	NT	870
	Lymph nodes of mice challenged with TSA-TRAIL cells	C3H	98	NT	880
	Spleen of virgin mice	BALB/c	427	33	40
	Spleen of mice challenged with TSA-pc	BALB/c	3041	39	36
	Spleen of mice challenged with TSA-TRAIL cells	BALB/c	495	30	33
	Spleen of virgin mice	C3H	231	NT	1321
	Spleen of mice challenged with TSA-pc	C3H	244	NT	1628
	Spleen of mice challenged with TSA-TRAIL cells	C3H	212	NT	1570

[a] Two weeks after 1×10^6 TSA-TRAIL or TSA-pc (H-2^d) challenge, inguinal lymph node cells (Expts. 1 and 2) and nylon wool purified spleen cells (Expt. 2) from pools of three B6 (H-2^b) mice were restimulated in vitro for 5 days with mitomycin-C-treated BALB/c (H-2^d) and C3H (H-2^k) spleen cells, and their lytic activity was tested in a 4-h ^{51}Cr release assay.

NT, not tested.

TSA-pc rejection in B6 mice was evaluated 2 wk after tumor challenge. Rejection of TSA-pc cells primed a strong lytic activity against H-2^d cells. That of TSA-TRAIL did not. The similar cytolytic response to H-2^k cells of mice that rejected TSA-pc and TSA-TRAIL suggests that a nonspecific immunosuppression due to factors released by TSA-TRAIL is not the reason for the diminished reaction to H-2^d cells (Table IV).

Inhibition of T lymphocyte reactivity by TSA-TRAIL cells

The ability of TSA-TRAIL cells to escape lysis by alloactivated T lymphocytes was evaluated in 4- and 48-h cytotoxicity assays. DBA/2 and B6 T lymphocytes were first stimulated with spleen cells from allogeneic mice. B6 anti-BALB/c lymphocytes killed TSA-pc moderately well in a 4-h ^{51}Cr release assay, and much better in a 48-h [^{3}H]TdR release assay. TSA-TRAIL cells were more resistant to lysis in both assays. In the 48-h assay, they were killed 90% less than TSA-pc (Table V).

Next, B6 anti-BALB/c lymphocytes stimulated by BALB/c spleen cells for 3 days in MLR were made to interact overnight over monolayers of TSA-pc and TSA-TRAIL cells in the presence of medium only, anti-TRAIL blocking IgG, ZVAD-fmk and Ac-YVAD-cmk inhibitors of caspase-1 (23), or 1% DMSO as control. In fact, TRAIL uses prototype caspases for intracellular signal transduction (24). TUNEL analysis showed that the percentage of apoptotic cells among the lymphocytes recovered was markedly higher following interaction with TSA-TRAIL than TSA-pc monolayers. Apoptosis was inhibited by the presence of both anti-TRAIL IgG and caspase-1 inhibitors (Fig. 4 and data not shown).

Discussion

The findings here reported suggest that the membrane expression of TRAIL allows cells to resist T cell reactivity both in vitro and in vivo. This resistance appears to be mainly due to TRAIL's ability to elicit apoptosis by interacting with activated T lymphocytes. Ex vivo morphological evaluations of TSA-TRAIL tumors show a much lower inflammatory and immune reaction than that elicited by TSA-pc. This was particularly noticeable following challenge in mice differing at multiple minor histocompatibility Ags, where the reactive cell subpopulations, especially T lymphocytes, were markedly reduced. The scanty infiltrate associated with a low production of cytokines was not in accordance with the expression of adhesion molecules by vascular endothelial cells and suggests that a fraction of reactive cells infiltrating the tumor via transendothelial migration is destroyed by TSA-TRAIL cells. Ultrastructural ex vivo data showing that lymphocytes interacting with TSA-TRAIL

Table V. *Different ability to kill TSA-TRAIL and TSA-pc (H-2^d) of DBA/2 (H-2^d) and B6 (H-2^b) lymphoid cells stimulated in MLR by BALB/c (H-2^d) or C3H (H-2^k) spleen cells*

Responder Cells	Stimulator Cells	LU_{20} 4-h ^{51}Cr release test TSA-pc	LU_{20} 4-h ^{51}Cr release test TSA-TRAIL	LU_{20} 4-h ^{51}Cr release test P815	LU_{20} [^{3}H]TdR release test TSA-pc	LU_{20} [^{3}H]TdR release test TSA-TRAIL
B6 lymph node cells	BALB/c	49	11	250	126	9
B6 spleen cells	BALB/c	83	44	404	765	73
B6 lymph node cells	C3H	16	0	9	0	2
B6 spleen cells	C3H	17	3	19	49	13
DBA/2 lymph node cells	BALB/c	14	4	0	11	0
DBA/2 spleen cells	BALB/c	23	19	18	55	24
DBA/2 lymph node cells	C3H	12	2	0	0	1
DBA/2 spleen cells	C3H	19	19	16	4	10

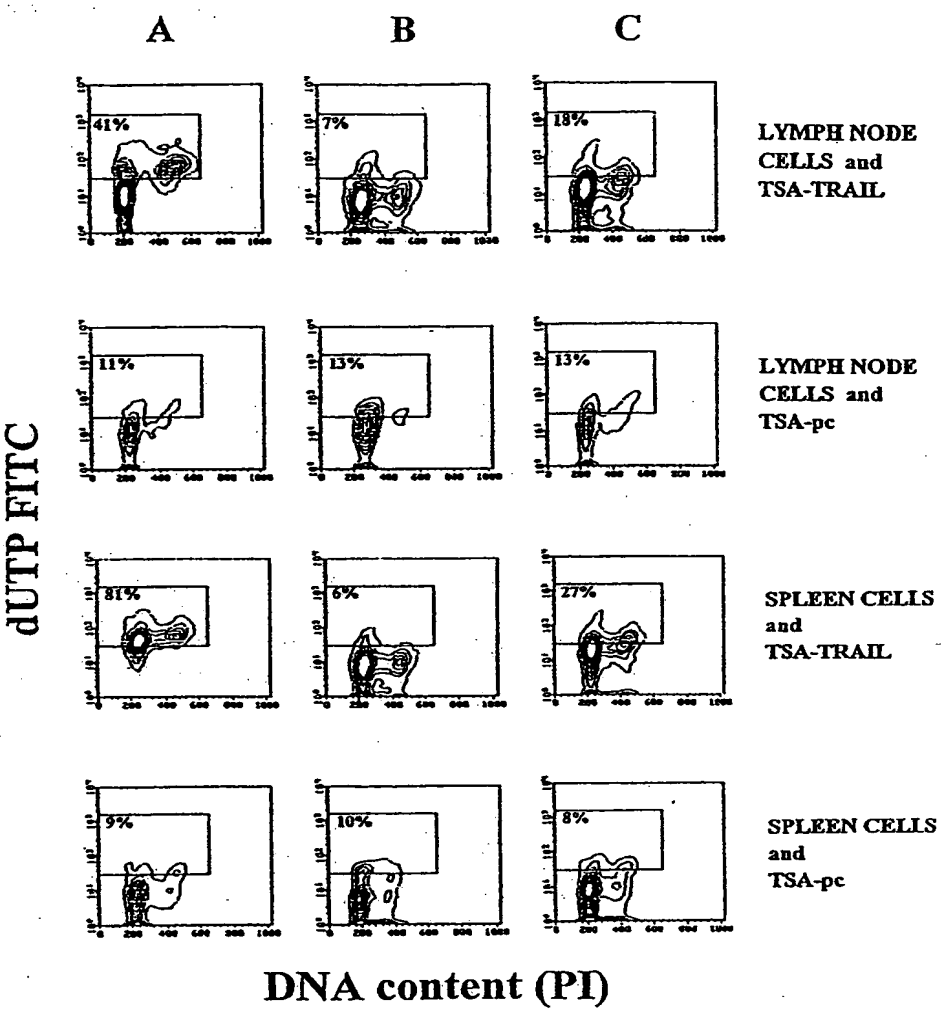

FIGURE 4. Apoptosis induced in B6 lymph node and spleen cells stimulated by BALB/c spleen cells in a 3-day MLR by 18-h interaction with TSA-pc and TSA-TRAIL monolayers in the presence of medium supplemented with 1% DMSO (column *A*), 50 μM of the inhibitor of caspase-1, ZVAD-fmk (column *B*), or 50 μg/ml of anti-TRAIL IgG (column *C*). Early apoptosis was evaluated by DNA strand break labeling with FITC-dUTP, as indicated by the region. Simultaneous staining of DNA with propidium iodide was performed. Flow cytometry revealed uptake of FITC-dUTP by apoptotic cells, as indicated by the region. FITC-dUTP negative/hypodiploid population indicating later apoptotic cells (37) was never detected. The corresponding region on the cytogram is not reported, and only the percentages of early apoptotic cells are shown. Inhibition of TRAIL-induced apoptosis was also observed when the 18-h interaction was done in the presence of 100 μM of the other inhibitor of caspase-1, Ac-YVAD-cmk: lymph node cells and TSA-TRAIL 15%; lymph node cells and TSA-pc 15%; spleen cells and TSA-TRAIL 12%; and spleen cells and TSA-pc 4%. The percentages of apoptotic cells in the presence of medium only are very similar to those obtained in the presence of 1% DMSO and are not shown, for the sake of simplicity.

cells undergo apoptosis endorse this possibility. Moreover, TSA-TRAIL cells fully or partially overcome the T lymphocyte-dependent tumor-specific immune memory elicited by preimmunization and alloreactions against non-H-2 or H-2 barriers. Elimination of reacting T lymphocytes probably underlies failure to boost alloreactivity.

Strong cytolytic responses directed to BALB/c histocompatibility Ags are poorly effective against TSA-TRAIL cells. Their resistance is particularly evident in the 48-h test, where a prolonged interaction between effector and TSA-TRAIL cells takes place. Moreover, an overnight interaction with TSA-TRAIL cells induces the apoptotic death of a significant number of specifically activated T lymphocytes. Apoptosis of reacting T cells appears to be directly due to the TRAIL on the membrane, since it is markedly diminished by anti-TRAIL IgG and by caspase-1 inhibitors. These findings, coupled with the evidence of apoptotic lymphocytes revealed by ultrastructural observations ex vivo, suggest that induction of apoptosis in activated lymphocytes that interact with TSA-TRAIL is an important mechanism by which they escape T cell reactivity. In effect, by interacting with a series of signaling receptors, TRAIL induces the apoptosis of many cells, including lymphocytes (9, 24–26). The apoptosis-inducing ability of TSA-TRAIL cells appears to be mainly due to their membrane-expressed TRAIL, since supernatants from TSA-TRAIL or TSA-pc cultures added to alloactivated lymphocytes do not influence their survival (data not shown).

A possible autocrine-positive loop in TSA-TRAIL cells and a direct survival advantage deriving from TRAIL membrane expression cannot be formally ruled out. However, the similarity between TSA-TRAIL and TSA-pc in their release of factors, MHC molecule expression, and growth pattern in vitro and in *nu/nu* mice seem to preclude a major effect of TRAIL on TSA cell behavior. The ability of TSA-TRAIL cells to evade strong T lymphocyte-dependent immune responses is somewhat similar to that of tumor cells that naturally express CD95L (14, 27–29). Their inability to elicit the neutrophil-dependent reaction leading to tumor rejection may be the key to the different in vivo behavior of CD95L and TRAIL-engineered cells (30, 31). CD95L, besides its ability to induce apoptosis of lymphoid cells and angiogenic activity (32), may well enhance endothelial cell expression of adhesion molecules and promote the neutrophil extravasation that leads to the rejection of tumor cells. However, immunohistochemical analysis revealed that TSA-TRAIL cells injected into syngeneic animals also activate endothelial cells, induce VCAM-1 and endothelial leukocyte adhesion molecule-1 expression, and favor development of the tumor vascular network. The accelerated rejection following CD95L transduction may rest on its quantitative expression (33). Overexpression may determine local endothelium activation (32)

strong enough to lead to marked neutrophil infiltration (30, 31). However, the different in vivo behavior of CD95L- and TRAIL-engineered tumors may be due to the diversities of these two ligands. TRAIL is 28% identical to CD95L and 23% identical to both TNF and lymphotoxin (1, 2) and acts on a different set of death-signaling receptors expressed by many normal and transformed cell types (4–8, 34). A complex modulation of multiple signaling and decoy receptors regulates the ability of TRAIL expressed by engineered tumors as well as by normal and neoplastic cells to suppress an immune response by delivering apoptotic signals (6, 11, 34). The susceptibility of lymphocytes to TRAIL-induced apoptosis changes in various situations (35–37). Data on the modulation of TRAIL receptor expression on lymphocyte activation are not yet available, and no correlation between receptor expression and the level of cell sensitivity to TRAIL apoptosis is evident (11, 34).

TRAIL-death receptors form a relatively newly characterized immune regulatory system, several aspects of which are not yet defined, though the present data offer an insight into its efficacy on genetically engineered tumor cells. Novel therapeutic strategies aiming to both hamper tumor escape from immune reactivity and facilitate allograft evasion from immune rejection should take it into serious account.

By contrast, the natural expression of TRAIL and other death ligands by tumor cells probably has a much less devastating inhibitory effect. Their expression is likely to be lower and extremely carefully regulated by cytokines and other environmental signals. Moreover, the presence of decoy receptors is another way to restrict their death potential. Cytokines regulating the expression of death ligands can also protect lymphocytes from tumor-induced apoptosis. These and other natural guards allowing lymphocytes to survive in the presence of TRAIL can also explain the rejection of TSA-TRAIL cells by fully allogeneic mice, despite the marked TRAIL expression on their cell membrane.

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